Signaling pathways involved in tumor necrosis factor α-induced upregulation of the taurine transporter in Caco-2 cells

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Received 10 March 2005; revised 5 April 2005; accepted 21 April 2005

Available online 5 May 2005

Edited by Frances Shannon

Abstract **We have previously demonstrated that the taurine uptake and transporter (TAUT) mRNA expression were upregulated by tumor necrosis factor α (TNF-α) in Caco-2 cells. In this present study, the signaling molecules related to this upregulation were investigated. Pyrrolidine dithiocarbamate, a nuclear factor κB (NF-κB) inhibitor, repressed the upregulation of taurine uptake and TAUT mRNA expression. A reporter assay revealed that TNF-α-induced TAUT transcriptional activity through the NF-κB consensus-like sequence in the human TAUT promoter region. An electrophoretic mobility shift assay showed that NF-κB could bind to the NF-κB consensus-like sequence. The anti-TNF receptor 1 (TNFR1) antibody, but not the TNF receptor 2 (TNFR2) antibody, repressed this upregulation.**

Keywords: Taurine; Transporter; Caco-2; Tumor necrosis factor α; Nuclear factor κB; TNF receptor

1. Introduction

Taurine (2-aminoethanesulfonic acid), a sulfur-containing β-amino acid, is the major free intracellular amino acid present in many tissues of mammals and plays such biological roles as detoxification, antioxidation, membrane stabilization and osmoregulation [1].

Taurine is transported by a specific transporter, the taurine transporter (TAUT). Most tissues are known to express TAUT, including the intestines. We have demonstrated that TAUT was regulated or modulated by changes in the extracellular osmolarity and taurine concentration of food-derived substances in human intestinal epithelial Caco-2 cells [2–4]. Furthermore, we have found that tumor necrosis factor α (TNF-α), one of the most prominent inflammatory mediators and central to the start of inflammatory reactions involving the innate immune system [5], increased the taurine uptake and taurine transporter (TAUT) mRNA expression in Caco-2 cells [6]. Two studies have also shown a TNF-α-induced increase in taurine uptake in cells derived from other tissues [7,8]. However, the signaling pathways involved in the TNF-α-induced upregulation of taurine uptake in these cells remain to be elucidated.

The signals from TNF-α have been well investigated. TNF-α specifically binds to TNF receptors 1 (TNFR1) and 2 (TNFR2) [5]. They share structural similarities in their extracellular domains, consisting of cysteine-rich repeats [9]. TNFR1 and TNFR2 differ from each other in their intracellular domains. TNFR1, but not TNFR2, has a 'death domain' (DD) in its intracellular make up. Since a TNF-α-induced apoptotic signal is transduced by DD containing adaptor molecules with the subsequent activation of caspases, it is not certain whether or not TNFR2 directly signals apoptosis. However, some reports have shown the activation of nuclear factor κB (NF-κB), p38 kinase or c-Jun N-terminal kinase to be mediated by TNFR2 [10–12].

Two types of signals are transduced via TNFR1: the apoptotic signal just described and an anti-apoptotic signal [13,14]. The NF-κB pathway is known as an anti-apoptotic signaling pathway. After binding TNF-α to TNFR1, this receptor complex is internalized [15], and the TNF receptor-associated death domain protein (TRADD) is recruited. TNFR1-bound TRADD can recruit an inhibitor of cellular apoptosis (cIAP) [16] and receptor-interacting protein (RIP), which enable binding of TNFR-associated factor 2 (TRAF2) to the TNFR1 signaling complex [11]. This complex results in the activation of different kinases such as NF-κB-inducing kinase (NIK) and IκB kinase (IKK) [17]. Activated IKK phosphorylates the IκBα protein on Ser^{32} and Ser^{36} [18], leading to ubiquitination on Lys^{21} and Lys^{22} with subsequent proteasome-dependent degradation [19]. The degradation and dissociation of IκBα from NF-κB leads to the nuclear transfer of NF-κB, and subsequent gene expression is initiated [20,21]. The genes downstream of NF-κB are related to inflammation and anti-apoptosis, including cIAP [13]. TNF-α also activates such MAP kinases as p38 and c-Jun N-terminal kinase (JNK) via TNFR1 [11,12,22].

Based on the information just presented, we investigated which signaling pathway was involved in the TNF-α-induced upregulation of taurine uptake in Caco-2 cells by using inhibitors of signal transduction.

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Abbreviations: cIAP, inhibitor of cellular apoptosis; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; HBSS, Hanks' balanced salt solution; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; LLnL, *N*-acetyl-leu-leu-norleucinal; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; RIP, receptor-interacting protein; SDS, sodium dodecyl sulfate; TAUT, taurine transporter; TLCK, tosyl-lys-chloromethylketone; TNF-α, tumor necrosis factor α; TNFR1, TNF receptor 1; TNFR2, TNF receptor 2; TRADD, TNF receptor-associated death domain protein; TRAF2, TNFR-associated factor 2

0014-5793/$30.00
doi:10.1016/j.febslet.2005.04.063

2. Materials and methods

2.1. Materials

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), pyrrolidine dithiocarbamate (PDTC), tosyl-lys-chloromethylketone (TLCK), genistein and *N*-acetyl-leu-leu-norleucinal (LLnL) were purchased from Sigma (St. Louis, MO, USA). Penicillin–streptomycin (10 000 U/ml and 10 mg/ml in 0.9% sodium chloride, respectively) and non-essential amino acids were purchased from Gibco (Gaithersburg, MD, USA). [1,2-^{3}H]taurine (specific radioactivity of 29 Ci/mmol), [γ-^{32}P]ATP and T4 polynucleotide kinase were from Amersham (Little Chalfont, Bucks., UK). Monoclonal anti-human TNFR1 was from HyCult Biotechnology (The Netherlands), monoclonal anti-human TNFR2 was from GT (Minneapolis, MN, USA), and human TNF-α was from Pepro Tech (Rocky Hill, NJ, USA), all the other chemicals being of reagent grade.

2.2. Cell culture

Caco-2 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air with a culture medium consisting of DMEM, 10% fetal calf serum, 1% non-essential amino acids, 2% glutamine, 200 U/ml of penicillin, 200 μg/ml of streptomycin and an appropriate amount of sodium bicarbonate. The cells were cultured at a density of 1.4×10^5 cells/well in 24-well plates that had been precoated with collagen and were then used for the uptake experiments.

2.3. Uptake experiments

The Caco-2 cells were washed twice with 700 μl each of Hanks' balanced salt solution (HBSS) containing 4 mM sodium bicarbonate and 10 mM HEPES, the pH value being adjusted to 7.4 with potassium hydroxide (the uptake buffer). The cells were then incubated at 37 °C for 10 min with 0.3 μCi of [^{3}H]taurine in 300 μl of the uptake buffer. At the end of the incubation period, the buffer was removed, and each monolayer was carefully washed twice for 5 min each with ice-cold phosphate-buffered saline (PBS) containing 0.05% sodium azide. To each well in the culture plate was then added 250 μl of 0.1% Triton X-100, before the dissolved cells were taken into 3 ml of a scintillation cocktail. The tritium content of each monolayer was finally determined with an LSC 5100 liquid scintillation analyzer (Aloka, Tokyo, Japan).

2.4. cDNA synthesis

RNA was isolated from the Caco-2 cells by using ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The RNA was reverse transcribed to cDNA by using the oligo dT primer and SuperScript™ II reverse transcriptase (Invitrogen, CA, USA) according to the manufacturer's instructions.

2.5. Primers

Specific primers for hTAUT and β-actin were purchased from Nihon Gene Research Laboratories (Sendai, Japan): forward primer for hTAUT, 5′-GAAATCTTCATCGCCTTCG-3′; reverse primer for hTAUT, 5′-ATAGCCAATCATGTCCTCA-3′; forward primer for β-actin, 5′-CCACGAAACTACCTTCAAC-3′; reverse primer for β-actin, 5′-GATCTTCATTGTGCTGGG-3′.

2.6. Quantification of PCR products

Human TAUT mRNA was quantified by using triplicate of samples, the housekeeping gene, β-actin, being used as a reference gene for quantification. Light Cycler PCR was performed with a DNA Sybr Green kit according to the manufacturer's instructions (Roche Diagnostics). Amplification was carried out for 40 cycles, each consisting of 15 s at 95 °C, 15 s at 57 °C and 25 s at 72 °C.

2.7. Western blot analysis

A cell extract was prepared as previously described [23], the extract being boiled and fractionated by SDS–PAGE (12.5%). The primary and secondary antibodies used in the experiments were, respectively, the rabbit anti-IκBα antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the donkey anti-rabbit IgG antibody linked to horseradish peroxidase (Amersham Biosciences). Proteins on the membrane were visualized by an enhanced chemi-luminescent method with an appropriate kit (Amersham Biosciences). NIH image software was used for the densitometric analysis.

2.8. Electrophoretic mobility shift assay

The cells were rinsed with ice-cold PBS without Mg^{2+} or Ca^{2+} (PBS(−)), and nuclear extracts were prepared as described previously [24,25]. An oligonucleotide probe containing the sequence of the NF-κB-binding site (sense, 5′-agcttCAGAGGGGACTTTCCGAGAGG-3′; antisense, 5′-tcgaCCTCTCGGAAAGTCCCCTCTGa-3′) labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase was incubated for 20 min with 5 μg of the nuclear extract at room temperature. The NF-κB bound to the labeled oligonucleotide probe was fractioned on 4% polyacrylamide gel and visualized by autoradiography. Double-stranded oligonucleotide composed of the 5′-GGAGGCTCCAGGGTCATTTCCTGAGA-3′ sequence, this being in the 5′-flanking region of the human TAUT gene and containing the NF-κB consensus-like sequence (nucleotides −2382 to −2372 of the human TAUT gene), was used for a binding and competitive assay. The NF-κB consensus-like sequence is underlined.

2.9. Construction of the reporter genes for a luciferase assay

The TAUT-NF-κB luciferase plasmid was constructed by inserting a tandem array of the NF-κB consensus-like sequence (CAAACGCGTCGCAGGGTCATTTCCTGAGCAGGGTCATTTCCTGAGCA GGGTCATTTCCTGAGCAGGGTCATTTCCTGAGGACAGATCTGCT) into the pGL3 promoter plasmid (Invitrogen, CA, USA).

2.10. Reporter assay

The reporter assay was performed as described previously [26–28]. Caco-2 cells were transfected with 3 μg of the indicated reporter construct and 0.1 μg of pRL-CMV, an expression plasmid encoding Renilla luciferase (Invitrogen), before being cultured for 24 h in a medium containing each indicated concentration of TNF-α. The Dual-Luciferase™ reporter system (Invitrogen) was used to determine the luciferase activity.

3. Results

3.1. Effects of the inhibitors of NF-κB on the TNF-α-induced upregulation of taurine uptake and TAUT mRNA expression by Caco-2 cells

Taurine uptake experiments were performed with Caco-2 cells which had been pretreated with an NF-κB inhibitor for 2 h and then precultured in a medium containing TNF-α and the inhibitor. PDTC, the pyrrolidine derivative of dithiocarbamate, belongs to a family of metal chelators with antioxidative properties that has been shown to inhibit NF-κB activation in various cell types, including Jurkat T cells, pre-B cells and human monocytes [19,29,30]. LLnL is a proteasome inhibitor that has been shown to block the breakdown of the inhibitor of NF-κB, IκB [31]. TLCK is a serine protease inhibitor with chymotrypsin-like specificity that has been shown to inhibit the activation of NF-κB in a variety of cell types, including Caco-2 cells [32]. Genistein inhibits tyrosine kinase activity [33] and has been shown to inhibit the activation of NF-κB in different cell types [34]. PDTC repressed the TNF-α-induced upregulation of taurine uptake in a concentration-dependent manner, while all the other inhibitors significantly repressed the upregulation(Fig. 1). The effect of PDTC on the increased TAUT mRNA caused by TNF-α was also examined by real-time PCR. RNA extracted from Caco-2 cells that had been cultured in the medium with TNF-α and PDTC for 24 h was reverse-transcribed to cDNA and used for real-time PCR. Although the cells treated with PDTC exhibited increased

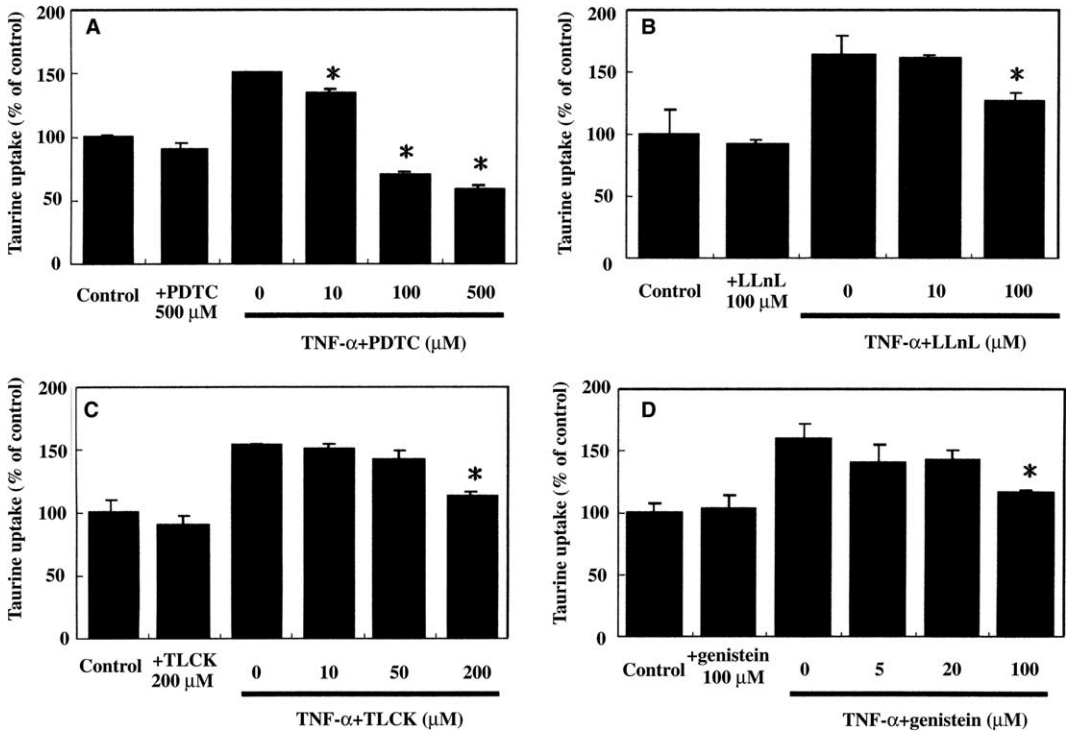

Fig. 1. Effect of PDTC, LLnL, TLCK or genistein on the TNF-α-induced upregulation of taurine uptake in Caco-2 monolayers. Caco-2 cells were pretreated with PDTC (0–500 μM; A), LLnL (0–100 μM; B), TLCK (0–200 μM; C) or genistein (0–100 μM; D) for 2 h before the cells were cultured for 24 h in a medium containing TNF-α (50 ng/ml) and each inhibitor. Uptake experiments were then performed as described in Section 2. These data are from a representative experiment of two independent experiments performed in quadruplicate. Each value is the mean ± S.E.M. $^{*}P < 0.05$ vs. the TNF-treated group without inhibitors by ANOVA.

TAUT mRNA expression, the increase by TNF-α was significantly suppressed by PDTC (Fig. 2).

3.2. Effect of PDTC on the TNF-α-induced IκBα degradation in Caco-2 cells

NF-κB in most cells is retained in the cytoplasm as an inactive tertiary complex with inhibitory protein known as IκBs. The degradation and dissociation of IκBα leads to the activation of NF-κB as described in Section 1. Therefore, the effect of PDTC, a potent inhibitor of NF-κB, on the TNF-α-induced IκBα degradation was examined. PDTC was found to retard the TNF-α-induced IκBα degradation in Caco-2 cells (Fig. 3), suggesting that PDTC inhibited the NF-κB activation in Caco-2 cells.

3.3. Contribution of the NF-κB consensus-like sequence in the promoter region of the human TAUT gene to transcriptional activation of the TAUT gene

We investigated whether activated NF-κB promoted transcriptional activity of the human TAUT gene through the NF-κB consensus-like sequence. Luciferase assays were performed with the TAUT-NF-κB luciferase plasmid as described in Section 2. TNF-α significantly increased the luciferase activity in Caco-2 cells (Fig. 4), suggesting that the TNF-α-induced

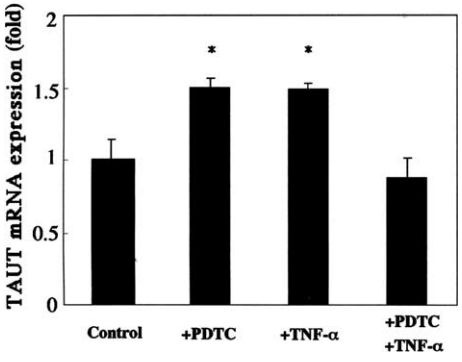

Fig. 2. Real-time PCR analysis of TAUT mRNA from Caco-2 cells cultured for 24 h in a medium containing TNF-α (50 ng/ml) and PDTC (100 μM). β-Actin was used as the control. These data are from a representative experiment of two independent experiments performed in triplicate. Each value is the mean ± S.E.M. $^{*}P < 0.05$ vs. the control by ANOVA.

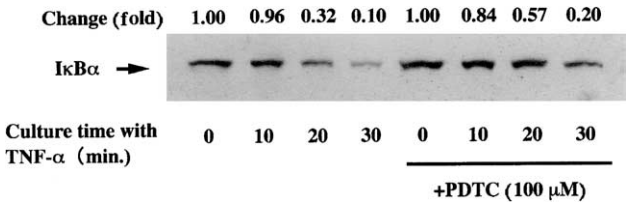

Fig. 3. Inhibition of PDTC to TNF-α-induced IκB degradation in Caco-2 cells. After being precultured in a medium containing PDTC for 1 h, the cells were stimulated by TNF-α (50 ng/ml) for the indicated times. Each cell extract (40 μg of protein) was fractionated by SDS–PAGE and blotted with anti-IκBα. These data are from a representative experiment of three independent experiments.

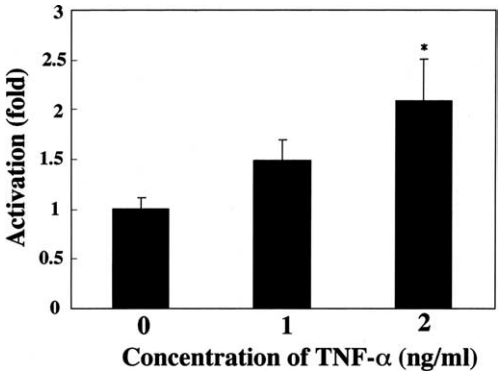

Fig. 4. Activation of the NF-κB consensus-like sequence of the TAUT promoter by TNF-α. The reporter assay was performed with the TAUT-NF-κB luciferase plasmid as described in Section 2. These data are from a representative experiment of three independent experiments performed in triplicate. Each value is the mean ± S.E.M. $^{*}P < 0.05$ vs. the control by ANOVA.

transcriptional activity of the human TAUT gene was upregulated through the NF-κB consensus-like sequence.

An electrophoretic mobility shift assay (EMSA) was performed to elucidate whether or not NF-κB was bound to the double-stranded oligonucleotide containing the NF-κB consensus-like sequence in the promoter region of the human TAUT gene. An oligonucleotide with the NF-κB-binding site was used as a probe. Binding of NF-κB to the probe was increased when Caco-2 cells were cultured with TNF-α for 12 or 24 h (Fig. 5). Competitive assays were performed by using the oligonucleotide containing the NF-κB consensus-like sequence as an unlabeled competitor. The competitor effectively inhibited NF-κB-probe-binding, suggesting that the NF-κB consensus-like sequence was able to bind to NF-κB.

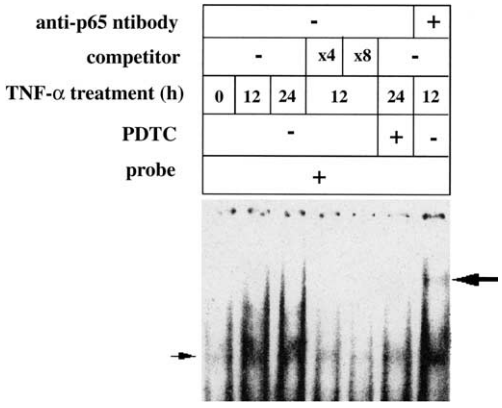

Fig. 5. TNF-α-promoted NF-κB binding to the NF-κB consensus-like sequence in the 5′-flanking region of the human TAUT gene. An EMSA was performed as described in Section 2. A 4- or 8-fold molar excess of unlabeled double-stranded DNA fragments derived from the 5′-flanking region of the human TAUT gene (see Section 2) was added in the competitive assays. The anti-p65 antibody was added in the supershift assay. The arrow on the left indicates the specific band and the thick arrow on the right indicates the supershift band. These data are from a representative experiment of three independent experiments.

3.4. Effects of anti-TNF receptor antibodies on the TNF-α-induced upregulation of taurine uptake by Caco-2 cells

TNF-α transduces signals via specific receptors, and two TNF-receptor (TNFR) isotypes, TNFR1 and TNFR2, are known to exist. RT-PCR was performed to confirm the expression of these receptors in Caco-2 cells. The PCR products were sequenced and identified them as TNFR1 and TNFR2 (data not shown). Uptake experiments were then performed by using each anti-TNFR antibody. When Caco-2 cells were incubated in a medium containing TNF-α and the anti-TNFR1 antibody, the TNF-α-induced upregulation of taurine uptake in Caco-2 cells was significantly repressed (Fig. 6A); however, the anti-TNFR2 antibody did not repress this upregulation (Fig. 6B), suggesting that TNFR1, but not TNFR2, was involved in the regulation.

4. Discussion

We investigated in this study the signaling molecules involved in the TNF-α-induced upregulation of TAUT in Caco-2 cells. The results show that NF-κB and TNFR1 were both involved in this regulation. Parikh et al. [35] have reported that the interleukin-1β (IL-1β)-induced interleukin-6 (IL-6) production in Caco-2 cells was inhibited by PDTC. Moon et al. [36] have reported that complementary C3 production in IL-1β-stimulated Caco-2 cells was blocked by PDTC. These findings suggest that the NF-κB activation in Caco-2 cells was suppressed by PDTC. We therefore examined the effect of PDTC on the TNF-α-induced upregulation of taurine uptake by Caco-2 cells. PDTC markedly repressed this upregulation in a concentration-dependent manner (Fig. 1). PDTC also repressed the TNF-α-induced increase in human TAUT mRNA expression (Fig. 2). In spite of the marked repressive effect of PDTC on TAUT mRNA expression, the results of the present study indicate that PDTC itself increased the TAUT mRNA expression (Fig. 2). PDTC is known to have metal-chelating and antioxidative properties which may explain the effect on Caco-2 cell functions and eventually on their TAUT mRNA expression. Other inhibitors that are known to inhibit NF-κB activation were therefore used to confirm the involvement of NF-κB in the taurine uptake experiments.

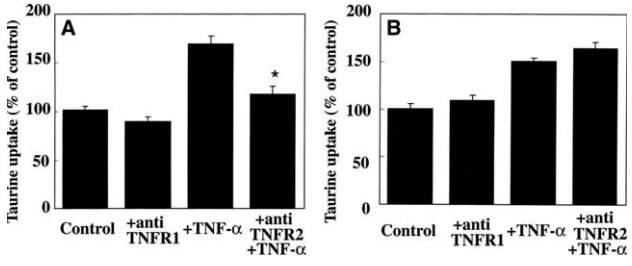

Fig. 6. Effects of antibodies against TNF receptors on the TNF-α-induced upregulation of taurine uptake by Caco-2 monolayers. Caco-2 cells were precultured for 24 h in a medium containing TNF-α (50 ng/ml) and the anti-TNFR1 antibody (10 μg/ml; A) or anti-TNFR2 antibody (50 μg/ml; B). Uptake experiments were then performed as described in Section 2. These data are from a representative experiment of two independent experiments performed in triplicate (A) and in quadruplicate (B). Each value is the mean ± S.E.M. $^{*}P < 0.05$ vs. the TNF-treated group by ANOVA.

Although each inhibitor is known to have some biochemical effects on the cells, besides inhibiting NF-κB activation, all the inhibitors used in the experiments significantly repressed the upregulation of taurine uptake (Fig. 1), strongly suggesting that NF-κB was involved in the regulation. PDTC retarded the TNF-α-induced IκBα degradation (Fig. 3) and inhibited the translocation of NF-κB activated by TNF-α to the nucleus (Fig. 5) in Caco-2 cells. Furthermore, as shown in Fig. 4, the transcriptional activity of the human TAUT gene could be activated through the NF-κB consensus-like sequence. These results suggest that repression of the TNF-α-induced increase in TAUT mRNA expression by PDTC was, at least partially, due to inhibited NF-κB activation. We also investigated this effect with inhibitors of other signal transduction molecules. PD98059 (a MEK inhibitor), SB203580 (a p38 inhibitor) and wortmannin (a PI3 kinase inhibitor) were used for the taurine uptake experiment. However, none of these inhibitors repressed the TNF-α-induced upregulation of taurine uptake (data not shown), suggesting that these signaling molecules were not involved in this regulation.

We confirmed that both TNFR1 and TNFR2 were expressed in Caco-2 cells and showed that TNFR1, but not TNFR2, mediated the signal (Fig. 6). However, factors which mediate the signal between TNFR1 and NF-κB have not yet been investigated. One possible mechanism could involve the activation of NF-κB by the signaling molecules that are recruited to TNF-α-bound TNFR1 (TRADD, RIP, TRAF2, etc.) as described in Section 1. Another possible mechanism could be that TNF-α promotes the production of reactive oxygen species in the cells and that the resulting oxidative stress activates NF-κB [37–39]. Further work will be needed to reveal details of such mechanisms.

It is interesting and worthwhile to consider the physiological meaning of the upregulation of taurine uptake in Caco-2 cells. We want to pay particular attention to the fact that taurine is an anti-inflammatory factor in intestinal epithelial cells. Being always exposed to a variety of xenobiotics, the intestines have defence systems to respond to these substances, the intestinal immune system being one such. Since this immune system is frequently activated, the intestines remain in a state of inflammation, although the level of such inflammation is normally controlled to be not excessive; for example, $CD4^+$ $CD25^+$ regulatory T cells (Tr) control the intestinal inflammation induced by both innate and adaptive immune responses via IL-10- and TGF-β-dependent mechanisms and have been shown to prevent a number of inflammatory diseases [40]. We have shown in this study that NF-κB activated by TNF-α could regulate TAUT gene expression at the transcriptional level. It has been reported that NF-κB transcriptionally upregulated such pro-inflammatory cytokines as IL-1β, IL-6 and IL-8 in intestinal epithelial cells and promoted inflammation[41]. On the other hand, we have previously found that taurine ameliorated TNF-α-induced Caco-2 cell damage [42]. Taking these findings into consideration, it is speculated that intestinal epithelial cells can show two different responses to TNF-α: one to promote inflammation by producing pro-inflammatory cytokines and the other to protect themselves from inflammatory damage by increasing the intracellular taurine concentration. NF-κB is likely to play a crucial role in this regulation by transcriptionally regulating not only pro-inflammatory cytokines, but also TAUT expression to control intestinal inflammation.

Acknowledgments: This study was supported by grant-in-aid for creative scientific research by the Japan Society for the Promotion of Science (No. 13GS0015). The uptake experiments in this work were performed at the Biotechnology Research Center of the University of Tokyo (Tokyo, Japan).

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